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SOIL MICROBIOLOGY

Does the Aboveground Herbivore Assemblage Influence Soil Bacterial Community Composition and Richness in Subalpine Grasslands?

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Abstract Grassland ecosystems support large communities of aboveground herbivores that are known to directly and indirectly affect belowground properties such as the microbial community composition, richness, or biomass. Even though multiple species of functionally different herbivores coexist in grassland ecosystems, most studies have only considered the impact of a single group, i.e., large ungulates (mostly domestic livestock) on microbial communities. Thus, we investigated how the exclusion of four groups of functionally different herbivores affects bacterial community composition, richness, and biomass in two vegetation types with different grazing histories. We progressively excluded large, medium, and small mammals as well as invertebrate herbivores using exclosures at 18 subalpine grassland sites (9 per vegetation type). We assessed the bacterial community composition using terminal restriction fragment length polymorphism (T-RFLP) at each site and exclosure type during three consecutive growing seasons (2009–2011) for rhizosphere and mineral soil separately. In addition, we determined microbial biomass carbon (MBC), root biomass, plant carbon:nitrogen ratio, soil temperature, and soil moisture. Even though several of these variables were affected by herbivore exclusion and vegetation type, against our expectations, bacterial community composition, richness, or MBC were not. Yet, bacterial communities strongly differed between the three growing seasons as well as to some extent between our study sites. Thus, our study indicates that the spatiotemporal variability in soil microclimate has much stronger effects on the soil bacterial communities than the grazing regime or the composition of the vegetation in this high-elevation ecosystem.

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Introduction

Grasslands cover approximately one third of the terrestrial landscape [1] and support a large community of aboveground vertebrate and invertebrate herbivores [2]. Herbivores can, in turn, be key determinants and regulators of grassland processes: besides altering aboveground biomass and plant species composition [e.g., 3–7], grazing by herbivores can directly and indirectly affect belowground properties. Direct impacts such as trampling can alter soil structure and permeability (e.g., bulk density or aeration; [8]), whereas the deposition of organic matter and nutrients in the form of dung and urine may stimulate root and microbial activity [9]. Thus, nitrogen (N) availability may be enhanced as nutrients ingested by herbivores are directly returned to the soil, "short-cutting" the nutrient cycle [10]. In addition, removal of aboveground biomass can indirectly lead to alterations in soil temperature or soil moisture [9, 11], which in turn can influence microbial activity and therefore decomposition of organic material. Herbivory can also alter physiological properties of plants such as C allocation between shoots and roots or the flow of C from the roots into the soil (root exudation; [11, 12]), which in turn

can stimulate microbial biomass and activity in the rhizosphere [13].

Furthermore, over longer time frames, herbivores can influence plant community composition by altering competitive interactions between plant species. This generally leads to an increase in the abundance of plant species with better defense against herbivory, e.g., to plant communities dominated by species with increased fiber or secondary metabolite content. As a consequence, the organic material entering the soil subsystem is of poorer resource quality, which retards soil processes [e.g., mineralization; 10, 11]. Additionally, it has been shown that the effect of herbivores on grassland properties highly depends on the grazing intensity (plant biomass consumption) as well as the productivity of the system [11]. Generally, it has been suggested that grazers have a positive effect on soil biota and soil processes in productive ecosystems in combination with high grazing intensities, while a negative effect is expected in systems with low productivity [see 11].

Consequently, the direct and indirect top-down effects on plants outlined above can induce bottom-up feedback-loops, since microorganisms are involved in a wide variety of ecosystem processes (e.g., nutrient mineralization). Thus, to understand feedback mechanisms within grassland ecosystems, it is important to link aboveground with belowground properties [11]. Many studies have assessed direct and indirect impacts of aboveground herbivores on soil processes (e.g., nutrient cycling: [14, 15]; decomposition: [9, 16]) or described the relation between grazing animals and microbes by investigating shifts in bacteria: fungi ratios [17–19]. Considerably, less is known on how herbivores affect bacterial community composition or richness. From the few conducted studies, it can be hypothesized that grazing can induce changes in bacteria functional groups [10, 20–22]. However, most studies only considered the impact of a single functional group of herbivores (usually large ungulates or domestic livestock [7, 10, 21], seldom other herbivores such as grasshoppers [23]) on microbial communities even though several species, groups, or taxa of functionally different herbivores of various body sizes generally coexist in a grassland ecosystem.

Given this lack of information, the objective of this study was to investigate how the exclusion of four groups of functionally different herbivores affects bacterial community composition, richness, and microbial biomass carbon (MBC) in two vegetation types that feature different grazing histories (and productivity) in the Swiss Alps—historically heavily grazed, rather nutrient-rich short-grass vegetation (further referred to as "short-grass vegetation" and historically lightly grazed, poorer quality tall-grass vegetation ("tall-grass vegetation"). More specifically, we investigated how one, two, and three growing seasons of progressive exclusion of large, medium, and small mammals and invertebrates affected the community composition and richness of bacteria in the rhizosphere and mineral soil using T-RFLP analyses as well as MBC (as a proxy for abundance) in these two vegetation types. In addition, we assessed how differences in bacterial parameters were related to abiotic and biotic variables (soil temperature, soil moisture, root biomass, and plant tissue C:N ratios). We expected distinct differences in bacterial community composition, richness, and MBC between the two vegetation types and greater shifts due to the exclusion of larger compared to smaller herbivores. This expectation was based on the fact that larger herbivores recycle more plant material through consumption and defecation compared to the smaller ones. We expected the differences in all variables to be more pronounced in the short-grass vegetation, as this vegetation type is generally grazed with greater intensity.

Material and Methods

Study Area

The study was conducted in the Swiss National Park (SNP) located in the southeastern Alps of Switzerland. Forests cover 50 km^2 of the park, grasslands 36 km^2, while the other half of the park area is covered with unproductive screed slopes, cliffs, and perpetual snow. Elevation ranges from 1,400 to 3,174 m above sea level. Founded in 1914, the SNP promotes a functionally diverse and easily observable grazer community since human disturbance is minimized (no hunting, fishing, or camping; visitors are not allowed to leave the trails). Mean annual temperature in the study region is 0.6±0.6 °C and mean annual precipitation is 871±156 mm (mean ± SD; recorded at the park's weather station in Buffalora, 1,977 m above sea level; [24]).

The subalpine grasslands are characterized by large homogeneous patches of short- and tall-grass vegetation. Short-grass patches are much smaller (up to 3 ha) and surrounded by extensive areas of tall-grass vegetation. Short-grass vegetation developed around former stables and huts where cattle and sheep were pastured at night (net nutrient import) during agricultural land-use (from fifteenth century until 1914). Tall-grass vegetation represents the areas where cattle and sheep used to graze during the day (net nutrient export) [25, 26]. Today, wild ungulates, medium and small mammals, as well as invertebrates graze on both vegetation types. The nutrient-rich short-grass vegetation is intensively grazed (up to 85 % of plant biomass consumed), while the tall-grass vegetation is characterized by significantly lower consumption rates (approximately 20 %; [26]), due to lower forage quality (lower phosphorus content [26] and N content [27]). Consequently, the average vegetation height of the short-grass vegetation typically ranges from 2 to 5 cm, and is dominated by red fescue (*Festuca rubra* L.), quaking grass (*Briza media* L.) and common bent grass (*Agrostis tenuis* Sipthrob). The tall-

grass vegetation exceeds 20 cm in height and is dominated by tussocks of evergreen sedge (*Carex sempervirens* Vill.) or mat grass (*Nardus stricta* L., [26]). The herbivore community grazing on these grasslands can be assigned to four functionally different groups also featuring different body masses: large [red deer (*Cervus elaphus* L.) and chamois (*Rupricapra rupricapra* L.); 30–150 kg], medium [marmot (*Marmota marmota* L.) and snow hare (*Lepus timidus* L.); 3–6 kg], and small vertebrate herbivores (small rodents: e.g., *Clethrionomys* spp., *Microtus* spp., *Apodemus* spp., 30–100 g) and invertebrate herbivores (mainly grasshoppers, aphids, thrips, and leafhoppers <5 g).

Experimental Design

Five exclosure treatments were set up in spring 2009 (immediately after snowmelt) at each of 18 sites in subalpine grasslands (9 on short-grass, 9 on tall-grass vegetation) ranging from 1,975 to 2,300 m above sea level. All grassland sites were located on dolomite parent material. These treatments consisted of five 2×3 m sized plots—four fenced plots that gradually excluded the different herbivore groups listed above—and a plot that was not fenced and thus accessible to all herbivore groups ("All" plot). This plot was located at least 5 m from the 2.1-m tall and 7×9-m sized main fence surrounding the other four plots. The fence was constructed of 10×10 cm wooden posts and electrical equestrian tape (AGRARO ECO, Landi, Bern, Switzerland; 20-mm width that was mounted at heights of 0.7, 0.95, 1.2, 1.5, and 2.1 m) connected to a solar-charged battery (AGRARO Sunpower S250, Landi, Bern, Switzerland). An additional tape was mounted at a height of 0.5 m, which was not connected to the power source, allowing marmots and hares to enter, while excluding deer and chamois. The "Marmot/Mice/Invertebrates" plot, located within this main fence, was left unprotected. Thus, with the exception of red deer and chamois, all other herbivore groups were able to access this plot. The fence around the 2×3 m "Mice/Invertebrates" plot consisted of a 90-cm high electric sheep fence (AGRARO Weidezaunnetz ECO, Landi, Bern, Switzerland; mesh size 10×10 cm) connected to the solar panel and excluded marmots and hares, while providing access for small mammals and invertebrates. The 2×3 m "Invertebrates" plot allowed access only for invertebrates and was surrounded by 1-m high metal mesh (Hortima AG, Hausen, Schweiz; mesh size 2×2 cm). The "None" plot was surrounded by a 1-m tall mosquito net (Sala Ferramenta AG, Biasca, Switzerland; mesh size 1.5×2 mm) to exclude all herbivores. The top of this plot was covered with a roof consisting of a 2×3 m wooden frame lined with mosquito mesh mounted on wooden corner posts. This plot was additionally treated with a biocompatible insecticide (Clean kill original, Eco Belle GmbH, Waldshut-Tiengen, Germany) when needed to remove insects that might have entered during data collection or that hatched from the soil. Fences were dismantled during winter (November to April) to protect them from snow pressure and avalanches and remounted in early May of each year immediately after snowmelt.

To assess whether the design of the "None" exclosure (mesh and roof) affected the microclimatic conditions, we erected "microclimate control" exclosures at 6 of the 18 sites. These exclosures were open at the bottom (20 cm) of the 3-m side to allow invertebrates to enter, but otherwise constructed as the "None" exclosures (for more details, see [28, 29]). Thus, this construction allowed a comparable microclimate to the "None" plots, but also a comparable grazing pressure to the "Invertebrates" plots. We compared various properties within these exclosures (Appendix Table A1) and were able to show that the exclusion of herbivores rather than the construction of our exclosures was responsible for the differences in parameters measured (see [28, 29]).

Biweekly ungulate pellet and grasshopper counts showed that all sites were grazed during all three years (Appendix Table A1; see Risch et al. [28] for more details). Marmot populations were counted (visual observation) twice during each summer, indicating that marmots were present at all sites (Appendix Table A1). Mice populations were not assessed, and no attempt was made to quantify herbivore numbers and composition within the individual exclosure networks. However, game cameras (Moultrie 6MP Game Spy I-60 Infrared Digital Game Camera, Moultrie Feeders, Alabaster, AL, USA) mounted at several fences showed that mice were present, that the medium- and small-sized mammals (marmots/hares and mice) were not afraid to enter the fences, and that they grazed on their "designated" plots. Our sites are grazed by each herbivore group with a consistent intensity from snow melt (early May) through late October (onset of snowfall), i.e., there is no "peak grazing period" as in other grassland ecosystems. Our herbivore exclusion treatments led to differences in consumption rates by the individual groups [27–29], which resulted in large differences in above- and belowground plant biomass. At the same time, our treatments also led to changes in feces/urine input by herbivores, although we could not measure these parameters on the individual plots (but see Appendix Table A1).

Soil Sampling and Belowground Measurements

At each site, soil sampling took place in early September of each growing season allowing for maximum herbivore impact before the vegetation becomes senescent. For this purpose, we selected one 1×1-m area within each of the five treatment plots. Within this area, two strips of 10 cm×1 m were cleared of vegetation, which was collected, dried at 65 °C, ground to pass a 0.5-mm sieve, and analyzed for plant tissue C and N concentration (Leco TruSpec Analyzer, Leco, St. Joseph, MI, USA).

Within these cleared strips, three soil samples were collected with a core sampler (5-cm diameter; AMS core sampler, AMS Inc, American Falls, ID, USA) at a randomly selected location. Then, two different soil layers were distinguished. In a first step, organic soil within the dense root layer (rhizosphere) was collected (typically 1 to 3 cm in depth), and in a second step, a 10-cm mineral soil core beneath the rhizosphere was taken. Due to the shallow soil depth in the study region, deeper soil sampling was not possible [30]. The three cores for each of the two layers were pooled and immediately stored in a cool-box. This sampling scheme amounted in a total of 540 soil samples (2 soil layers × 5 treatments × 18 exclosures × 3 years of sampling). Samples were sieved through a 2-mm sieve upon return to the field station. A subsample of soil (see below), glass beads, and extraction buffer were placed into an Eppendorf tube, briefly mixed on a shaker, and then frozen at −20 °C until further analyses were conducted.

To assess root biomass, five soil samples were randomly collected within the same strips as described above with a soil corer (2.2-cm diameter, Giddings Machine Company, Windsor, CO, USA) to a depth of 10 cm. Samples were dried at 30 °C and subsequently roots were manually separated from soil material. Each sample was sorted for an hour, which assured that over 95 % of all roots were picked. Before weighing to the nearest milligram, roots were dried at 65 °C for 48 h. The average data of the five samples was used to calculate root biomass per square meter.

Soil temperature and moisture were measured every other week throughout the field season (May–September) of each year. Measurements were conducted for the 0 to 12-cm depth at the previously assigned quadrat of each plot. Soil temperature was measured with a waterproof digital pocket thermometer (Barnstead International, Dubuque, IA, USA) and soil moisture was assessed at five random points within the quadrat with a FieldScout TDR-100 (time domain reflectometer; Spectrum Technologies, Plainfield, IL, USA).

Determining Bacterial Community Structure and Microbial Biomass Carbon

Bacterial community composition within the rhizosphere and mineral soil layers of each soil sample was analyzed following the protocol recommended by Frey et al. [31]. The 16S rDNA genes of the total extracted DNA were PCR-amplified and terminal restriction fragment length polymorphism (T-RFLP) was used to profile the bacterial community composition. For the DNA extraction, 350–600 mg soil material and 750-mg glass beads (0.1-mm diameter, B. Braun Biotech International, Melsungen, Germany) were suspended in 1.3-ml DNA extraction buffer (2 % CTAB, 20 mM EDTA pH 8, 2 M NaCl; 100 mM Tris THAM pH 8, 2 % PVP) and subsequently frozen until further treatment. Extraction was obtained using a bead beating procedure (FastPrep 120, Savant Instruments, NY, USA) of 40 s at 5.5 m s^{-1} followed by 5 min centrifugation at 13,000 rpm. Each soil sample was extracted three times with repeated addition of 1 ml of extraction buffer and pooled with its supernatant. The pooled extract of all three extraction steps was purified with 2 ml chloroform-isoamyl alcohol (24:1 *v*/*v*). Precipitation of DNA was achieved through the addition of 3 ml isopropanol incubated for 1 h at 37 °C, followed by a 15-min centrifugation at 13,000 rpm. The pellets were washed with 70 % EtOH, air dried and resuspended in 220 μl of AE buffer (10 mM Tris-Cl, 0.5 mM EDTA, pH 9).

To bind PCR inhibiting substances such as humic acids, samples were processed with a BSA-pretreatment: 12.5 μl AE buffer, 2.5 μl BSA (Fluka, Buchs, Switzerland), and 10 μl DNA were incubated for 5 min at 90 °C. Amplification of bacterial 16S rDNA gene was conducted with a PCR with a fluorescent-labeled (6-FAM) forward primer 27f (5′-AGAG TTTGATCMTGGCTCAG-3′) and an unlabeled reverse primer 1378r (5′-CGGTGTGTACAAGGCCCGGGAACG-3′). A 20-μl reaction mixture consisting of 0.2 μM of each of the primers, 1× PCR-buffer (QIAGEN, Hilden, Germany), 2 μM $MgCl_2$, 0.4 mM deoxynucleoside triphosphate (Promega), 0.6 mg ml^{-1} BSA, and 0.05 U/μl HotStar Taq polymerase (QIAGEN) was added to 5 μl of prediluted (1:50) DNA. PCR amplification was performed with a PTC-100 thermocycler (MJ Research, Waltham, MA, USA) with the following cycling conditions: an initial activating step for HotStar Taq polymerase (15 min at 95 °C) was followed by 35 amplification cycles consisting of 45 s denaturation at 95 °C, 45 s annealing at 48 °C, and 2 min extension at 72 °C. The PCR amplification was terminated with an additional 5 min final extension step at 72 °C. The amplification success was verified by electrophoresis on a 1 % agarose gel in 1 % TAE buffer.

The PCR product was digested using 0.1 U of the restriction nuclease *Msp*I (Promega) in 1 % Y Tango buffer diluted with HPLC water and incubated for 3 h at 37 °C, followed by a 20-min inactivation step at 65 °C. Digestion products were then desalted with Montage SEQ96 plates (Millipore Corporation, Billerica, MA, USA). For this execution, a vacuum (22–23 bar) was applied to let the digestion products, which were preliminarily transferred into the wells, flow through the membrane. The membrane was then washed twice with 20 μl of AE-buffer, applying the same procedure. After using another 20 μl AE-buffer incubated on a shaker for 10 min at 750 rpm, DNA was resuspended and transferred to a new PCR plate.

For the T-RFLP analysis, a mix of 1 μl of the previously obtained restriction digests, 0.1 μl of the internal size standard ROX500 (Applied Biosystems, Foster City, CA, USA), and 12.9 μl HiDi formamide (Applied Biosystems, Foster City, CA, USA) was denaturated for 5 min at 95 °C, and then chilled in ice water before separation using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City,

CA, USA). The analyzer was equipped with a 36 cm capillary, which was filled with POP-4™ polymer. T-RFLP profiles were analyzed using Genotyper v3.7 NT (Applied Biosystems, Foster City, CA, USA). Terminal restriction fragments (T-RFs) above a signal threshold of 50 relative fluorescence units were determined manually. Relative signal intensities were obtained by dividing signal intensities of each individual T-RF by the sum of all signal intensities of a sample. This norm compensates differences in PCR product quantity and T-RFLP fingerprint intensity among samples, which is necessary when signal intensities among different samples are compared [32]. It should be noted that not every terminal restriction fragment identified by the T-RFLP analysis necessarily represents a single species, since it is possible that different species have restriction fragments with exactly the same number of base pairs. Thus, we define the individual restriction fragments as operational taxonomic units (OTUs), which can be considered a conservative estimate of the number of bacterial species present in our system. We are aware that using only one restriction enzyme might have led to an underestimation of microbial richness as bacteria can share the same terminal restriction fragments for a particular primer. Yet, the method used allowed us to assess relative differences in bacterial community composition when aboveground herbivores were removed, which was our goal.

Microbial biomass C was determined using the substrate-induced method of Anderson and Domsch [33]; glucose (5 g kg^{-1} soil; the optimum concentration for CO_2 production in these soils) was added to 25 g of sieved mineral soil (dry weight equivalent), and CO_2 production was measured using a LI-COR 6200 gas analyzer (LI-COR Biosciences, Lincoln, NE, USA) 1–2 h after glucose addition. Prior to the analysis, samples were brought to 60 % water-filled pore space with addition of deionized water and were incubated for 10 days at room temperature to re-equilibrate microbial activity following disturbance due to sampling. This incubation time was identified by a preliminary experiment as the minimal time during which microbial activity reaches a steady state (basal respiration). Microbial biomass C was determined for mineral soil only as the rhizosphere samples were too small for analysis. Collecting additional samples was not feasible given the small size of the 1 × 1 m plots and an experiment that lasted 5 years in total.

Statistical Analysis

Nonmetric multidimensional scaling (NMDS) with subsequent linear mixed model analyses performed on the NMDS scores were used to test for variation in bacterial community composition based on presence-absence data of the rhizosphere and the mineral soil separately. The experimental factors of exclosure treatment (five levels; see experimental design), vegetation type (short-grass vs. tall-grass vegetation), year (2009, 2010, 2011), and all possible interactions were treated as fixed effects and site (fence) as a random factor. NMDS was based on Sørensen distances between samples (i.e., Bray-Curtis calculated on presence/absence data), 50 restarts, and a stability criterion of 0.001. We assessed the relationships of biotic and abiotic variables (soil temperature, soil moisture, root biomass, plant tissue C:N ratio, and MBC) as well as some geographical properties of the fences (elevation, north–south (NS) and east–west (EW) coordinates) with the axis scores of the three NMDS axes (axis1, axis2, and axis3) by correlation analyses (Pearson's correlation coefficient). In addition, we tested how site location affected the community composition by running one-way ANOVAs with NMDS axes scores as dependent variables and site (fence) as an independent factor.

Linear mixed model analysis was performed to analyze variation in OTU richness and MBC (square-root-transformed). The analysis of OTU richness was done separately for the rhizosphere and the mineral soil samples. The models comprised the same fixed and random effects as described above. Tukey's HSD post hoc tests with Bonferroni correction were used to test for significant differences between the herbivore exclusion treatments. As there were no significant treatment × year interactions, indicating that the effects of herbivore exclusion were not significantly different among the three years of the experiment, only the results of the overall model including the data of all years are reported. Indeed, results of separate analyses for each year were qualitatively identical with respect to herbivore exclusion effects. We also assessed which of the measured biotic and abiotic variables (soil temperature, soil moisture, root biomass (log-transformed), and plant tissue C:N ratio) were correlated with richness and mineral soil MBC (square-root-transformed) using Pearson correlation coefficients. In addition, we used linear mixed model analyses with the fixed and random effects mentioned above to assess how herbivore exclusion affected the biotic and abiotic variables. With the exception of the NMDS, which were performed with PRIMER 6 (PRIMER-E Ltd, Plymouth, UK), all statistical analyses were performed with the PASW Statistics 19.0 statistical package (IBM SPSS, Chicago, IL, USA).

Results

Bacterial Community Composition and Richness

Over the three years of the experiment, a total of 89 OTUs of different length (ribotypes) were detected. Fragment lengths ranged from 50.16 to 496.91 bp. The three-dimensional NMDS had a minimum stress-value of 0.124 for the rhizosphere (Fig. 1a) and 0.084 for the mineral soil bacterial communities (Fig. 2a). We found no evidence that herbivore

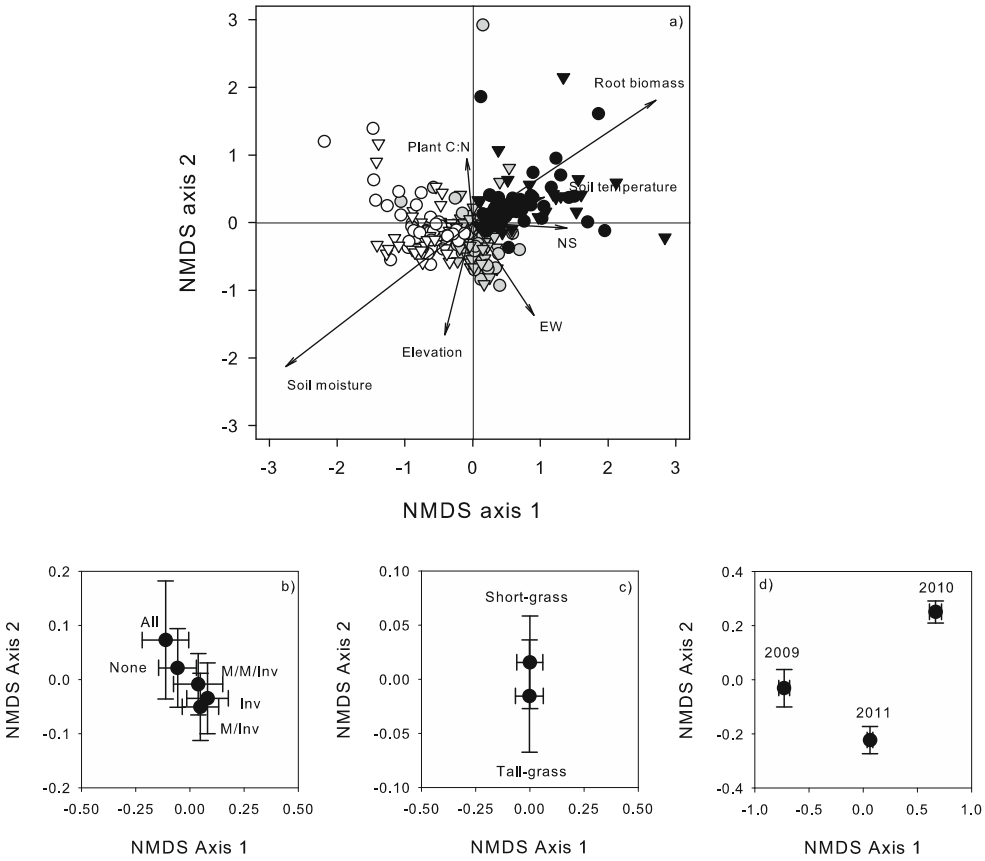

Fig. 1 Results of NMDS axes 1 and 2 for the rhizosphere soil samples. **a** All samples in relation to abiotic and biotic ecosystem parameters (*black arrows*). *NS* is the north–south coordinate of the exclosure network, *EW* is the east–west coordinate of the exclosure network. *Circles* represent short-grass vegetation, *triangles* represent tall-grass vegetation. *Gray filling* stands for the 2009, *black* for the 2010, and *no filling* for the 2011 growing season. **b** NMDS axes 1 and 2 mean ± standard error for the individual treatments (M/M/Inv = Marmot/Mice/Invertebrates, M/Inv. = Mice/Invertebrates, Inv. = Invertebrates), **c** NMDS axes 1 and 2 mean ± standard error for the two vegetation types, and **d** NMDS axes 1 and 2 mean ± standard error for the three growing seasons

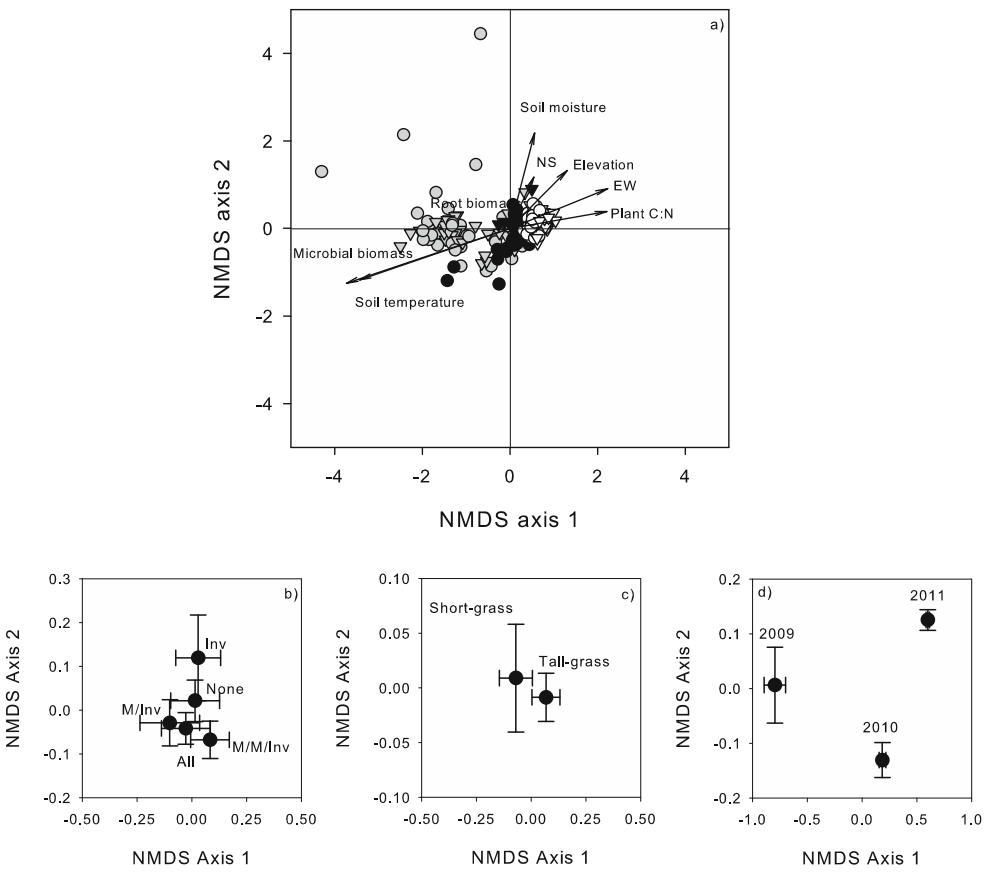

Fig. 2 Results NMDS axes 1 and 2 for the mineral soil samples. **a** All samples in relation to abiotic and biotic ecosystem parameters (*black arrows*). *NS* is the north–south coordinate of the exclosure network, *EW* is the east–west coordinate of the exclosure network. *Circles* represent short-grass vegetation, *triangles* represent tall-grass vegetation. *Gray* filling stands for the 2009, *black* for the 2010, and *no filling* for the 2011 growing season. **b** NMDS axes 1 and 2 mean ± standard error for the individual treatments (M/M/Inv = Marmot/Mice/Invertebrates, M/Inv. = Mice/Invertebrates, Inv. = Invertebrates), **c** NMDS axes 1 and 2 mean ± standard error for the two vegetation types, and **d** NMDS axes 1 and 2 mean ± standard error for the three growing seasons

exclusion treatments or vegetation type significantly affected the composition of bacterial communities in the rhizosphere or mineral soil (Table 1; Figs. 1b, c and 2b, c). However, interestingly, the bacterial community composition of both the rhizosphere and mineral soil significantly differed between the three years (Table 1; Figs. 1d and 2d). Differences in bacterial community composition in the rhizosphere soil showed strongest relations to soil moisture and root biomass (Fig. 1a; Table 2). The soil temperature, plant C:N concentrations, and geographic location somewhat affected the bacterial communities, but with regard to locations, we only detected differences between the fences on the second axis [NMDS axis 2 (results not shown); Table 2]. In contrast to the rhizosphere soil, strong relationships were found between the bacterial community composition and all variables except root biomass and north–south coordinates in the mineral soil (Fig. 2a; Table 2). Geographic location affected the bacterial community composition in the mineral soil somewhat more compared to the rhizosphere as we found differences between the 18 fences on 2 of the 3 NMDS axes (axes 1 and 3; results not shown).

OTU richness of neither the rhizosphere ($F_{4,239}$= 0.175, p=0.951) nor the mineral soil was significantly affected by herbivore exclusion ($F_{4,223}$=0.676, p=0.609). Moreover, vegetation type had no overall effect on this response variable in the rhizosphere ($F_{4,239}$=0.151, p=0.698) or the mineral soil ($F_{4,223}$=0.745, p=0.401). However, mineral soil bacterial richness strongly varied among the three growing seasons ($F_{4,223}$=145.926, p<0.001), whereas no among-year variation was detected for the rhizosphere ($F_{4,239}$=0.386, p=0.680; Appendix Table A2). Mineral soil bacterial richness was positively correlated with the biotic variables root biomass and plant C:N concentration and negatively with the abiotic variables soil temperature and soil moisture (Table 3). In contrast, no correlation was found between rhizosphere bacterial richness and these biotic or abiotic variables (Table 3).

Soil Microbial Biomass Carbon and Soil Properties

Mineral soil MBC significantly differed between the herbivore exclusion treatments varying between 765±47 (mean ± SE) and 973±56 mg/kg soil over the course of our study ($F_{4,\ 227}$= 3.579, p=0.007). It was lowest in plots from which ungulates and marmots ("Mice/Invertebrates") were excluded and highest in plots with free access for all but ungulates ("Marmot/Mice/Invertebrates"; Fig. 3). It should, however, be noted that the treatment differences were only (weakly) significant for 2010 ($F_{4,64}$=2.568, p=0.046), but not for the other two years of investigation (2009: $F_{4,63}$=1.052, p=0.387; 2011: $F_{4,64}$=1.535, p=0.203). MBC significantly differed between the three years of sampling with highest values in 2009 compared to the other two years (Appendix Table A2). No difference was found in MBC between the two vegetation types

Table 1 Statistical results from the linear mixed model approach applied on the NMDS axes scores of bacterial community structure data for both the rhizosphere and mineral soil

		Axis1		Axis2		Axis3	
	df	*F*	*p*	*F*	*p*	*F*	*p*
			Rhizosphere soil				
Treatment	4,223	2.170	0.073	0.532	0.712	0.444	0.777
Vegetation type	1,16	0.001	0.975	0.111	0.743	0.208	0.655
Year	2,223	274.9	<0.001	19.92	<0.001	138.8	<0.001
Treatment × vegetation type	4,223	0.960	0.430	0.832	0.506	0.567	0.687
Treatment × year	8,223	0.643	0.741	1.517	0.152	0.804	0.600
Vegetation type × year	2,223	0.660	0.518	0.062	0.939	0.162	0.851
Treatment × vegetation type × year	8,223	0.581	0.793	0.500	0.855	1.287	0.251
			Mineral soil				
Treatment	4,223	0.961	0.430	1.741	0.142	0.454	0.769
Vegetation type	1,16	1.084	0.313	0.086	0.773	0.660	0.429
Year	2,223	176.1	<0.001	8.668	<0.001	24.30	<0.001
Treatment × vegetation type	4,223	0.348	0.845	1.449	0.219	1.421	0.228
Treatment × year	8,223	0.891	0.525	1.253	0.270	1.203	0.298
Vegetation type × year	2,223	0.285	0.752	3.854	0.023	0.004	0.996
Treatment × vegetation type × year	8,223	0.211	0.989	0.864	0.548	0.784	0.617

df degrees of freedom, *F*=*F* value, *p*=*p* value

Table 2 Correlation between the three NMDS axes scores and abiotic and biotic parameters

	Rhizosphere soil							Mineral soil						
	Axis1			Axis2			Axis3				Axis2			Axis3		
	r	*p*	*n*	r	*p*	*n*	r	*p*	*n*	r	*p*	*n*	r	*p*	*n*
Micro biomass	NA	NA	NA	NA	NA	NA	NA	NA	NA	−0.366	<0.001	269	−0.116	0.058	269	0.145	0.018	269
Soil temp	0.106	0.082	269	0.038	0.534	269	0.239	<0.001	269	−0.343	<0.001	269	−0.188	0.053	269	−0.070	0.250	269
Soil moist	−0.276	<0.001	269	−0.213	<0.001	269	0.080	0.191	269	0.056	0.356	269	0.218	<0.001	269	0.012	0.849	269
C:N	−0.009	0.889	269	0.095	0.122	269	−0.191	0.002	269	0.222	<0.001	269	0.038	0.530	269	0.127	0.037	269
Root	0.250	<0.001	269	0.200	0.001	269	0.004	0.950	269	0.042	0.469	269	−0.013	0.835	269	−0.006	0.928	269
Elev	−0.041	0.501	269	−0.166	0.006	269	−0.037	0.546	269	0.131	0.032	269	0.133	0.029	269	−0.124	0.043	269
NS	0.139	0.023	269	−0.008	0.895	269	−0.141	0.021	269	0.055	0.371	269	0.118	0.054	269	−0.035	0.572	269
EW	0.090	0.140	269	−0.137	0.024	269	−0.181	0.003	269	0.223	<0.001	269	0.092	0.134	269	−0.216	<0.001	269

Soil temp soil temperature in °C, *soil moist* soil moisture (%), *Elev* elevation (m), *NS* north–south coordinate, *EW* east–west coordinate, *micro biomass* microbial biomass mg/kg, *Root* root biomass (g/m^2), *C:N* plant material C:N ratio, *r* Pearson correlation coefficient, *p*=*p* value, *n*=number of data points

Table 3 Correlations between bacterial community richness and abiotic (soil temperature, soil moisture) and biotic (root biomass, plant C:N ratio) parameters for both the rhizosphere and mineral soil separately

	Rhizosphere soil			Mineral soil		
	Richness			Richness		
	r	*p*	*n*	r	*p*	*n*
Root biomass	0.039	0.522	269	0.180	0.003	269
Soil temperature	−0.024	0.701	269	−0.218	<0.001	269
Soil moisture	0.042	0.494	269	−0.139	0.022	269
Plant C:N	−0.067	0.271	269	0.203	0.001	269

r Pearson correlation coefficient, *p*=*p* value, *n*=number of data points

(short-grass: 817±31, tall-grass: 921±38 g/kg; $F_{1,16}$=0.638, p=0.436). Generally, mineral soil MBC was positively related to soil temperature (r=0.233, p<0.001; n=269), and negatively to both soil moisture (r=− 0.219, p<0.001; n=269) and plant tissue C:N ratio (r=−0.129, p=0.034; n=269), but not to root biomass (r=0.077, p=0.210; n=269).

Our progressive herbivore exclusion resulted in a reduction of soil temperature consistently across the two vegetation types ($F_{4,223}$=15.556, p<0.001). Moreover, there was a vegetation-type-dependent effect of herbivore exclusion on root biomass (significant treatment × vegetation type interaction: $F_{4,223}$=4.02, p=0.003): herbivore exclusion reduced root biomass only in the tall-grass vegetation ($F_{4,112}$=3.987, p=0.003). Soil moisture significantly differed between the herbivore treatments ($F_{4,223}$= 2.583, p=0.038). Similar as for root biomass, we found a

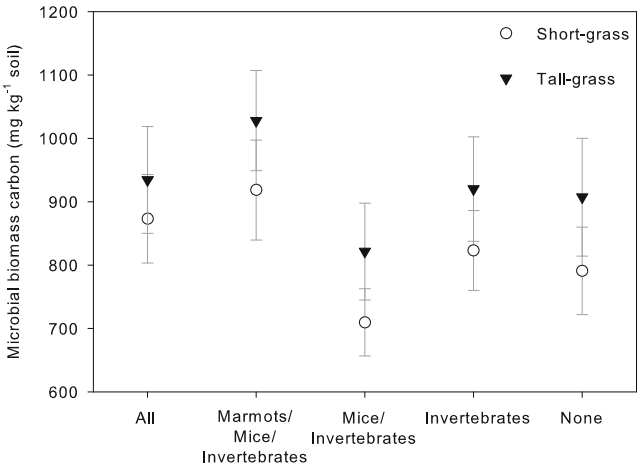

Fig. 3 Effect of herbivore removal on soil microbial biomass carbon (*MBC*) for the two different vegetation types. Values represent mean ± standard error. Labels on *x*-axis represent herbivore groups present

vegetation-type-dependent effect of herbivore exclusion on plant C:N ratios (treatment × vegetation type interaction: $F_{4,223}$=4.403, p=0.002), with herbivore exclusion decreasing plant tissue C:N ratio in the tall-grass vegetation only ($F_{4,122}$= 4.728, p=0.001). All biotic and abiotic variables significantly differed over the course of the three years (see Appendix Table A2).

Discussion

Bacterial Community Composition and Richness

Against our expectations, we did not detect an influence of herbivore exclusion on bacterial community composition. These findings contrast most studies that dealt with grazing effects (large ungulates) on soil bacterial communities: For example, Zhou et al. [7] detected differences in bacterial community structure in grasslands grazed by sheep at different intensities: their communities differed in areas with low to medium grazing intensities (1.33 and 4.0 sheep ha^{-1}, respectively) compared to ungrazed and heavily (6.67 sheep ha^{-1}) grazed sites. Also Attard et al. [34] and Clegg et al. [21] reported shifts in bacterial community structure caused by grazing in a microcosm and a field experiment, respectively. Both explained their findings by changes in the inputs of urine and feces, changes in plant composition, and soil structure. Also Grayston et al. [35] and Waldrop and Firestone [36] suggested that differences in plant community composition were responsible for resulting differences in bacterial community composition. In our study, we only found small changes in vegetation community composition by 2012. Haynes [27] showed that only 1 of 157 plant species—*F. rubra*—increased in cover, while more competitive plant species (according to Grimes' CSR strategies [37]) gained slightly higher cover compared to stress-tolerant species in both vegetation types. This lack in major plant community changes could explain the lack of differences in bacterial community composition between our treatments. Yet, we (i) considerably altered urine and feces inputs with our experimental setup (ungulate feces input see Appendix Table A1; no data available for the other species) and (ii) worked in two considerably different plant communities (for differences also see [25]). But, again, these differences did not result in changes in bacterial community structures either. We acknowledge that we cannot exclude that our treatments might have affected other groups such as Archaea and fungi. Yet, this would need further research.

The only differences that we detected were temporal, i.e., between the three growing seasons (interannual) of sampling and partly spatial, i.e., between the different sites. These findings suggest that interannual and spatial variability in air temperature and precipitation (data not shown) led to variations in soil temperature and soil moisture, which likely also affected the amount and quality of available resources for the bacterial community. Several authors reported seasonal as well as interannual patterns in microbial community composition [e.g., 35, 36, 38]. They attributed their findings to temporal variation soil temperature, soil moisture, and resource availability. Our study suggests a similar mechanism.

Microbial Biomass Carbon and Soil Properties

Excluding functionally different herbivore groups from subalpine grasslands only resulted in slight differences in mineral soil MBC in 2010, when MBC variability was lowest, but not during the other years. Thus, the data should be interpreted with care. Generally, authors explained decreases in MBC due to grazing exclusion by a reduction in nutrient input via dung and urine and decreases in biomass consumption rates [e.g., 10, 16, 39]. We do have large decreases in the input of large ungulate dung (and also urine), but do not have data for our plots. It is therefore difficult to assess the specific effect of dung and urine reduction on MBC in detail. Again, we did not detect any differences in MBC between our two vegetation types and the strongest differences in MBC were among the three growing seasons. Thus, our MBC results contrast with the findings of Bardgett et al. [10] who reported higher microbial biomass in *Nardus* dominated vegetation—similar to our tall-grass—compared to *Agrostis-Festuca* vegetation that is similar to our short-grass. Yet, in their study, the two grassland types were located on different parent material that featured different pH, which was not the case in our study. Interannual differences in MBC have often been reported from semiarid grasslands [40, 41] and a tall-grass prairie [42], where soil moisture was positively related with MBC. This is apparently in contrast to the negative correlation of soil moisture with MBC that we found in our study. However, the grassland ecosystems mentioned above are likely moisture limited compared to our high-elevation, alpine ecosystems that are temperature limited. Risch et al. [28] and Haynes et al. [29] indeed showed that soil moisture was negatively related to soil temperature in our study system, with increases in moisture and decreases in temperature when herbivores were progressively excluded.

Lack of Response of Soil Bacterial Community Composition and Richness to Herbivore Removal and Grazing History

Excluding the different herbivores for three growing seasons from two different grassland types did, against our expectations, not result in changes of the bacterial community composition and richness, even though our treatments had large effects on above- and belowground biomass [results; 28, 29] and likely also feces/urine input by herbivores. A potential explanation for this lack in response could be that the time of exclusion was too short to allow for changes. This argument would be supported by the fact that many studies that reported grazing-related differences in bacterial community composition were performed over fairly long time frames (e.g., 16 years [7]; 37 years [10]), during which the ecosystems had time to adjust to the new grazing regimes and ecosystem properties were likely altered.

Yet, we also did not detect any differences in bacterial community composition and richness between the two vegetation types with different plant communities and ecosystem properties that were grazed at different intensities for decades. This indicates that the bacterial communities present in our system respond either very slowly to changes in grazing regimes and the concomitant changes in the vegetation composition, or that other factors than these alterations of ecosystem parameters are more important in our high-elevation grasslands. A very slow response is certainly possible, as already discussed above. Yet, our findings suggest that it is more likely that the spatiotemporal variability in the soil microclimate had a stronger effect on ecosystem properties than the alterations in these variables caused by herbivory or plant community type. This would be in line with the findings by Frank and Esper [43] and Cannone et al. [44] that suggested that high-elevation ecosystems are more sensitive to variability in climate parameters compared to ecosystems at lower elevations.

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Appendix

Table 4 Average number of ungulate pellet groups 100 m^{-2}, number of marmots counted, and average number of grasshoppers m^{-2} at each site for summer 2009 and 2010

		Pellet groups 100 m^{-2}			Marmots $site^{-1}$			Grasshoppers m^{-2}		
Site	Vegetation type	2009	2010	2011	2009	2010	2011	2009	2010	2011
1	Short-grass	13.7	12.4	9.4	5.0	1.0	4.0	0.5	5.3	0.8
2	Tall-grass	6.0	8.3	7.7	5.0	1.0	4.0	0.7	5.0	0.7
3	Short-grass	9.3	12.1	9.4	5.0	1.0	4.0	0.5	1.5	0.6
4	Tall-grass	7.7	10.1	8.9	5.0	1.0	4.0	0.9	5.9	1.3
5	Short-grass	17.8	11.9	12.1	3.0	2.0	4.0	0.6	2.7	0.9
6	Tall-grass	8.3	8.5	4.4	3.0	2.0	4.0	0.9	3.8	0.4
7	Short-grass	1.8	8.9	6.1	9.0	1.0	4.0	0.2	0.4	0.3
8	Tall-grass	15.2	10.6	12.0	9.0	1.0	4.0	0.6	1.6	0.1
9	Short-grass	1.7	4.4	3.4	2.0	2.0	3.0	0.1	1.0	0.4
10	Tall-grass	6.0	9.4	4.9	2.0	2.0	3.0	0.2	0.3	0.1
11	Short-grass	13.2	13.2	11.6	17.0	21.0	16.0	0.5	2.8	0.5
12	Tall-grass	15.2	21.0	16.3	17.0	21.0	16.0	1.6	4.4	1.4
13	Short-grass	17.3	15.0	15.3	17.0	21.0	16.0	1.1	3.6	0.7
14	Tall-grass	26.0	13.6	12.5	17.0	21.0	16.0	0.8	3.5	1.4
15	Short-grass	20.3	6.9	7.8	11.0	11.0	9.0	0.5	0.4	0.2
16	Tall-grass	9.2	6.5	4.6	11.0	11.0	9.0	0.2	0.1	0.1
17	Short-grass	28.3	14.2	11.6	11.0	11.0	9.0	0.3	0.7	0.1
18	Tall-grass	10.5	5.6	3.8	11.0	11.0	9.0	0.3	0.3	0.2

Table 5 Interannual differences in bacterial community richness for both the mineral soil and the rhizosphere. Interannual differences in environmental variables microbial biomass (MBC; mg/kg), soil temperature in °C; soil moisture (%), plant material C:N ratio and root biomass (g/m^2); values represent mean ± standard error. F=F value, p=p value; different letters indicate significantly different values

	2009	2010	2011	F	p
Richness (mineral)	49.3±1.70b	73.4±0.92a	70.2±0.65a	123.49	<0.001
Richness (rhizosphere)	70.0±0.70	69.3±1.01	68.8±0.97	0.430	0.651
Microbial biomass	1061.4±45.8a	764.3±35.1b	795.3±37.0b	16.73	<0.001
Soil temperature	13.7±0.16a	13.0±0.16b	12.5±0.16c	12.53	<0.001
Soil moisture	34.8±1.44a	26.7±0.83b	35.8±0.82a	22.12	<0.001
Plant tissue C:N	24.2±0.30b	26.5±0.34a	26.7±0.40a	14.98	<0.001
Root biomass	688.4±37.42b	972.4±38.87a	627.2±19.36b	31.16	<0.001

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